Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/002543

International filing date: 27 January 2005 (27.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/540,864
Filing date: 30 January 2004 (30.01.2004)

Date of receipt at the International Bureau: 31 March 2005 (31.03.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

1297599

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APPLICATION NUMBER: *60/540,864*
FILING DATE: *January 30, 2004*
RELATED PCT APPLICATION NUMBER: *PCT/US05/02543*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 305 642 398 US

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TITLE OF THE INVENTION (500 characters max)

C-TERMINAL p-53 PALINDROMIC PEPTIDE THAT INDUCES APOPTOSIS OF CELLS WITH ABERRANT p53 AND USES THEREOF

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 28
☑ Drawing(s) *Number of Sheets* 8
☐ Application Date Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.
☑ A check or money order is enclosed to cover the filing fees.
☑ The Director is herby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 03-3125
☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.
☑ Yes, the name of the U.S. Government agency and the Government contract number are: National Science Foundation grants R01 OH07590 and R01 CA82528

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Date January 30, 2004

REGISTRATION NO. 39,992 *(if appropriate)*

Docket Number: 0575/68074-PRO/JPW

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
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PTO/SB/16 (08-03)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Docket Number 0575/68074-PRO/JPW

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Docket No.: 68074-PRO/JPW/GJG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Robert L. Fine et al.

Serial No.: Not Yet Known

Filed: Herewith

For: C-TERMINAL p53 PALINDROMIC PEPTIDE THAT INDUCES APOPTOSIS OF CELLS WITH ABERRANT p53 AND USES THEREOF

1185 Avenue of the Americas
New York, New York 10036
January 30, 2004

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C-TERMINAL p53 PALINDROMIC PEPTIDE THAT INDUCES APOPTOSIS OF CELLS WITH ABERRANT p53 AND USES THEREOF

This invention has been made with government support under National Science Foundation grants R01 OH07590, and R01 CA82528. Accordingly, the U.S. Government has certain rights in the invention.

Throughout this application, various publications are referenced within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Background of the Invention

More than 50% of human malignancies, including breast cancers, are associated with missense mutations or deletions of p53, and most of the missense mutations map to the DNA-binding domain of the protein. The nucleotide sequence of the human p53 gene and the amino acid sequence of the encoded p53 protein have been reported (Zakut-Houri et al. (1985), EMBO J., 4: 1251-1255; GenBank Code Hsp53). The p53 protein consists of 393 amino acids and its functional domains have been characterized (e.g., U.S. Patent No. 6,326,464).

p53 is a sequence-specific transcriptional factor that transactivates a number of genes whose products are involved in cell growth regulation. These include WAF1/p21/Cip1, which arrests the cell cycle, GADD45 for DNA repair, and Bax and Fas/APO-1 to modulate apoptosis. Apoptosis is a complex process regulated by several pathways, some of which involve members of the Bcl-2 family and the Fas pathway. Wild-type p53 forms a

tetramer to perform its tumor suppressor activity.

The sequence-specific DNA-binding activity of p53 appears to be negatively regulated by its C-terminal 30-amino acid (aa) segment (aa 363-393) and also by N-terminal proline-rich motifs located between aa 80-93. Synthetic peptides corresponding to the C-terminal domain of p53 such as residues aa 363-393 bind directly in vitro to over-expressed wild-type and mutant p53. Binding experiments with p53 proteins that contain selected deletions indicate that binding of the free aa 363-393 peptide to p53 requires the presence of both C-terminal aa 363-393 and N-terminal aa 80-93 sequences in the p53 protein. This observation suggests either that the free peptide may interact simultaneously with both regions or that the absence of either or both of these segments in p53 results in structural changes in the protein, lowering its affinity for the free peptide.

Deletion of either or both of these regulatory regions, as well as various C-terminal modifications, stimulate specific DNA binding of p53 *in vitro*. Previous studies demonstrate that the addition of a chemically modified C-terminal p53 peptide restored *in vitro* sequence-specific DNA binding function to mutant p53-273 (Arg to His). Furthermore, intranuclear microinjection of this peptide into SW480 colon carcinoma cells carrying an endogenous p53-273 His mutation restored transcriptional activation of a p53-responsive reporter construct.

It was previously demonstrated that a synthetic peptide derived from the C-terminal regulatory region of the p53 tumor suppressor protein (amino acids 361-382) could induce p53-dependent, apoptotic cell death in the presence of mutant p53 with minimal effect on cells with normal

levels of wild-type 53 (A.L. Kim et al., *Conformational and molecular basis for induction of apoptosis by a C-terminal peptide in human cancer cells*, J. Biol. Chem. (1999) 274:34924-34931). Since p53 mutations occur in over 50% of common human cancers, a therapy directed at specifically killing p53-mutant cells would have wide application.

Summary of the Invention

An embodiment of the inventions is a polypeptide comprising a first segment of continuous amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1) covalently linked to a second segment of continuous amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2).

Another embodiment of the invention is a polypeptide comprising at least two covalently linked segments of continuous amino acids, each segment comprising consecutive amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1), or consecutive amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2).

A further embodiment of the inventions is a nucleic acid comprising nucleotides encoding any of the disclosed polypeptides, and a plasmid which expresses any of the disclosed polypeptides.

Yet another embodiment of the invention is a method of killing cancer cells that contain mutant p53 or over-expressed wild-type p53 by contacting the cancer cells with the disclosed polypeptides, and a method of treating a subject suffering from cancer by administering to the subject the disclosed polypeptides.

Detailed Description of the Figures

Figure 1

Construction of C-Terminal p53 palindromic tetrapeptide.

Figure 2

Purification of bacterial lysate produced His-tagged-p53 4-repeat-Ant carrier peptide. Bacterial lysates were first applied to the Nickel-nitrilotriactic column. The histidine amino acids of the 6-His tag bind to the nickel ions immobilized by the nitrilotriactic groups on the agarose matrix. The column is then washed with low concentrations of imidazole (25 mM) to remove nonspecific, low affinity endogenous bacterial proteins (not shown). Increasing the concentration of imidazole from 50 mM (E1), to 100 mM (E2), to 150 mM (E3), and finally to 250 mM (E4), displaces the histidine from the nickel ions so that the tagged protein is removed. Then acrylamide displaces the histidine from the nickel ions so that the tagged protein is removed. This acrylamide (12%)/SDS PAGE gel was stained with 0.25% Ponceau Red solution. STD-protein standards.

Figure 3

Effects of the C-Terminal p53 Palindromic Tetrapeptide on Cells in Culture. MDA-MB-468 human breast cancer cells were grown in culture and exposed for 6 hours with no peptide (control) or with 30 μM of either the single p53 C-terminal peptide fused to the Ant carrier (p53-Ant) or with the His tagged p53 palindromic tetrapeptide fused to the Ant carrier (4 Repeat). The DNA within the cells was then stained with propidium iodide and cell particles were assayed by flow cytometry. The percent within each graph represents the percent cell particles with less than a diploid amount of DNA, indicative of apoptotic cells (sub

G1 population).

Figure 4

Effects of endogenous, regulated expression of the 4 repeat peptide on cell viability. MDA-MB-468 cells engineered to express the palindromic p53 4 repeat peptide under the control of a tetracycline responsive promoter were cultured without or with the addition of 2 μg/ml doxycycline, an analog of tetracycline, for 24, 48 or 72 hours. Cells were collected, fixed in ice cold 70% ethanol and the DNA was stained with propidium iodide. Cell cycle profiles were obtained by flow cytometry and the percent of sub-G1 cell particles, indicative of apoptosis, was quantified.

Figure 5

Changes in the protein expression levels for the pro-apoptotic protein Bax, an active p53 responsive gene, induced by exposure to the tetracycline analog doxycycline (2 μg/ml) for 12, 36 and 48 hours. Engineered MDA-MB-468 cells were collected after the indicated exposure times and the total protein was extracted, an aliquot was run on a 12% acrylamide/SDS PAGE gel, and blotted onto nitrocellulose membranes. Antibodies specific for Bax, the 4-repeat (p53) and PARP were used to determine expression levels and α-tubulin was used as a loading control. A PARP cleavage product, indicative for apoptosis, is observed even 12 hours after 4-repeat induction and expression. Of note, the 4 repeat (C-terminal palindromic tetrapeptide) induced Bax while our previous studies have shown that the monomer, p53 peptide-Ant, did not.

Figure 6

Changes in the protein expression levels for the p53

responsive genes encoding Fas and caspase 8, 24 hours after the engineered MDA-MB-468 cells were exposed to the indicated concentrations of doxycycline. A PARP cleavage product is also shown. α-tubulin was used as a loading control.

Figure 7

TUNEL assay on engineered MDA-MB-468 human breast cancer cells after 24 hours of exposure to 2 µg/ml Doxycycline. Regulated expression of the p53 4 repeat peptide results in the endonuclease cleavage of chromatin DNA, as seen as a shift from no doxycycline (control) to 24 hours after Doxycycline (Dox 2 µg/ml). If the caspase 8 specific inhibitor (IETD-FMK) at 2 µM is added 6 hours after doxycycline is added, then no shift occurs 18 hours later (24 hr total doxycycline treatment).

Figure 8

Propidium labeled DNA, indicative of apoptosis, induced by the monomer p53 peptide-Ant and the tetrapeptide (4 repeat)±Ant. Constructs of the plasmids were transfected into DU-145 human prostate cancer cells, which carry an endogenous mutant p53, by adenovirus vector using a multiplicity of infection (MOI) of 50. In addition, these plasmids encoded green fluorescent protein (GFP) as a way to check for successful transfection into the DV-145 cells. The only cells which died were those which received the 4 repeat (C-terminal palindromic tetrapeptide) in their construct with or without Ant. Thus, the short monomeric p53 peptide-Ant was proteolyzed too quickly for its activity to manifest while the 4 repeat exhibited stability and thus induced cell death.

Detailed Description of the Invention

An embodiment of the inventions is a polypeptide comprising a first segment of continuous amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1) covalently linked to a second segment of continuous amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2).

The polypeptide may further comprise a glycine between the first segment and the second segment

In an embodiment, the polypeptide may comprise amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPG ETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 3), wherein the glycine may be present or absent.

In another embodiment, the polypeptide may comprise amino acids having the palindromic sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGK EPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 4), wherein the glycine may be present or absent.

In yet another embodiment, the polypeptide may comprise amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPG ETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKEPGGS RAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPGETKFMLKKHR STSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 5), wherein the glycine may be present or absent.

In a further embodiment, the polypeptide may comprise amino acids having the palindromic sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGK

EPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPGETKFM LKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKEPGGSRAHSS HLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 6), wherein the glycine may be present or absent.

In yet a further embodiment, the polypeptide may comprise amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSDDSDPGETKFMLKKH RSTSQGKKSKLHSSHARSGGPEKGAQAAQAGKEPGGSRAHSSHLKSKKGQSTSRH KKLMFKTEGPDSDDSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 7).

In another embodiment of the invention, the polypeptide comprises at least two covalently linked segments of continuous amino acids, each segment comprising consecutive amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1), or consecutive amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2). The polypeptide of this embodiment can contain three or four of the segments covalently linked.

Any of the described polypeptides may further comprise a six repeat histidine tag attached to the N-terminus of the polypeptide.

Any of the described polypeptides may further comprise a membrane carrier peptide attached to the C-terminus of the polypeptide. The membrane carrier peptide may comprise amino acids having the sequence KKWKMRRNQFWVKVQRG (SEQ ID NO. 8).

Any of the described polypeptides may further comprise, together, a six repeat histidine tag attached to the N-terminus of the polypeptide, and a membrane carrier

peptide attached to the C-terminus of the polypeptide.

A further embodiment of the inventions is a nucleic acid comprising nucleotides encoding any of the disclosed polypeptides, and a plasmid which expresses any of the disclosed polypeptides.

Yet another embodiment of the invention is a method of killing cancer cells that contain mutant p53 or over-expressed wild-type p53 by contacting the cancer cells with the disclosed polypeptides, and a method of treating a subject suffering from cancer by administering to the subject the disclosed polypeptides.

The ability of the described polypeptides and compositions of this invention to activate the DNA binding activity of p53 and thus activate the cellular functions of p53, enables their use as pharmaceutical compositions in a variety of therapeutic regimens. The present invention therefore includes novel therapeutic pharmaceutical compositions and methods for treating a human or animal with such compositions. As used herein, the term "pharmaceutical" includes veterinary applications of the invention.

To prepare the pharmaceutical compositions of the present invention, at least one polypeptide, or alternatively, a mixture of polypeptides of this invention is combined as the active ingredient in admixture with a pharmaceutical carrier selected and prepared according to conventional pharmaceutical compounding techniques. This carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral, sublingual, rectal, nasal, parenteral intraperitoneal, intravenous, intraarterial.

Pharmaceutically acceptable solid or liquid carriers or components which may be added to enhance or stabilize the composition, or to facilitate preparation of the composition include, without limitation, syrup, water, isotonic saline solution, 5% dextrose in water or buffered sodium or ammonium acetate solution, oils, glycerin, alcohols, flavoring agents, preservatives, coloring agents starches, sugars, diluents, granulating agents, lubricants, and binders, among others. The carrier may also include a sustained release material such as glyceryl monostearate or glyceryl distearate, alone or with a wax. The amount of solid carrier varies but, preferably will be between about 20 mg to about 1 g per dosage unit.

Pharmaceutical compositions of the peptides of this invention, or derivatives thereof, may therefore be formulated as solutions of lyophilized powders for parenteral administration. Another method of administration is that of intravenous or intraarterial administration.

Pharmaceutical compositions of this invention may also include topical formulations incorporated in a suitable base or vehicle, for application at the site of the area for the exertion of local action. Accordingly, such topical compositions include those forms in which the formulation is applied externally by direct contact with the skin surface to be treated. Conventional forms for this purpose include but are not limited to creams, ointments, lotions, gels, pastes, powders and formulations having oleaginous absorption, water-soluble, and emulsion-type bases.

Additionally, the compounds of the present invention may also be administered encapsulated in liposomes. The

compositions, depending upon its solubility, may be present both in the aqueous layer and in the lipidic layer, or in what is generally termed a liposomic suspension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such a diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature.

The compositions may be supplemented by active pharmaceutical ingredients, where desired. Optional antibacterial, antiseptic, and antioxidant agents may also be present in the compositions where they will perform their ordinary functions.

Dosage units of such pharmaceutical compositions containing the polypeptides of this invention preferably contain about 1 mg-5 g of the peptide or salt thereof.

As used herein, the terms "suitable amounts" or "therapeutically effective amount" means an amount which is effective to treat the conditions referred to below. A polypeptide of the present invention is generally effective when parenterally administered in amounts from 1 mg per kg of body weight to 100 mg/kg.

The pharmaceutical compositions described above and identified with the ability to activate the DNA binding activity of p53 are useful in therapeutic regimens which exploit the cellular functions of p53.

Compositions of this invention may be administered parenterally (for example, intravenously) as an adjunct to patients receiving traditional cancer therapy, which

employs the use of DNA damaging agents (eg. radiation therapy and chemotherapy). The compositions of this invention may also be employed as the sole treatment for patients with cancer to enhance the tumor suppressor function of p53, whether wild-type or mutant, present in tumor cells. The administration of the composition to a cancer patient thus permits the arrest of the growth or proliferation of tumor cells or apoptosis (cell death) of tumor cells. A suitable amount of the composition of this invention is administered systemically, or locally to the site of the tumor.

Definitions

A palindrome, or a palindromic sequence, is used herein to mean a polypeptide comprising amino acids having a sequence which is the same when read from the N-terminal to the C-terminal, as when read from the C-terminal to the N-terminal, e.g. "ABCBA" or "ABCCBA".

A "construct" is used to mean recombinant nucleic acid which may be a recombinant DNA or RNA molecule, that has been generated for the purpose of the expression of a specific nucleotide sequence(s), or is to be used in the construction of other recombinant nucleic acids. In general, "construct" is used herein to refer to an isolated, recombinant DNA or RNA molecule.

Viral vector is used herein to mean a vector that comprises all or parts of a viral genome which is capable of being introduced into cells and expressed. Such viral vectors may include native, mutant or recombinant viruses. Such viruses may have an RNA or DNA genome. Examples of suitable viral vectors include retroviral vectors (including lentiviral vectors) (WO 94/06910), adenoviral vectors (WO 94/24297), adeno-associated viral vectors and

hybrid vectors.

A retroviral vector is a viral vector where the virus is from the family retroviridae.

Transduction is used to refer to the introduction of genetic material into a cell by using a viral vector.

As used herein a transduced cell results from a transduction process and contains genetic material it did not contain before the transduction process, whether stably integrated into the genome or as an episome.

Transfection refers to the introduction of genetic material into a cell without using a viral vector. Examples of transfection include insertion of "naked" DNA or DNA in liposomes, that is without a viral coat or envelope.

The phrase "pharmaceutically acceptable carrier" is used to mean any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, and water.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intracerebral, intraspinal and intrasternal injection and infusion.

The preparations of the present invention may be given parenterally, orally topically, or rectally. They are of course given by forms suitable for each administration route. For example, they are administered by injection, orally inhalation, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories.

Details of general formulation procedures and information on additional excipients may be found in Remington: The Science and Practice of Pharmacy, 20th Edition.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

EXPERIMENTAL DETAILS

A. Materials and Methods

A series of primers were designed for PCR to gradually extend the C-terminal peptide codon sequence (amino acids 353-393) so that a second C-terminal peptide codon sequence would be conjugated in reverse to the original sequence; this palindromic sequence was engineered so that an additional codon for the amino acid glycine (CGG) occurred between the two sequences to insure maximum structural flexibility at the junction of the palindromes (i.e., amino acids 353-393-Gly-393-353). An additional codon for the amino acid glycine (CGG) was then inserted downstream of the 5'-terminal 353 alanine codon (GGC), which thereby generated a unique restriction enzyme site for MspI (GGCCGG (SEQ ID NO. 9)). This restriction enzyme site made possible the fusion of two of these dimer codon sequences together to form the codon sequence for a palindromic tetramer (Figure 1). An additional PCR with unique primers resulted in the insertion of a start methionine codon at the 5' end and a termination codon at the 3' end of the palindromic tetramer codon sequence. Furthermore, additional restriction enzyme sites were generated at both termini of the tetramer construct to facilitate proper expression orientation into any cloning or expression vector. The proper sequence of the final expression construct was confirmed by DNA sequence analysis.

For cloning, in order to generate sufficient peptide we selected a bacterial protein expression system (QIAexpressionist, Qiagen, Valencia, CA). All procedures were performed with materials supplied by the manufacturer according to their instructions. At the N-terminus of the tetramer construct a six repeat histidine tag was inserted

as part of the pQE plasmid. In addition, at the C-terminus the 17 amino acid coding sequence for the membrane carrier peptide derived from Antennaepedia was inserted to allow for free passage of the expressed peptide through cell membranes. Appropriate restriction enzyme sites (EcoRI at the 5' end and HindIII at the 3' end) were engineered to bracket the start codon-His tag-C-terminal p53 palindromic tetramer-Antennapedia carrier-stop codon sequence. Reconstruction of the supplied plasmid, using PCR with specially designed primers, was necessary to ensure that no additional codons were inserted at any junction point. The final sequence was confirmed by DNA sequence analysis (Figure 1).

Since this system is designed to produce high levels of the exogenous protein, the production of the product is regulated so that bacteria can replicate and cultures can be established. Once sufficient E. coli containing the construct has been cultured, isopropyl-(3-D-thiogalactoside (IPTG) is added to the cultures to stimulate exogenous protein production. IPTG binds to the trans-expressed lac repressor protein excoded by the lac I gene (a bacterial clone is provided as part of the system and is maintained by co-expression of a kanamycin resistance gene). Multiple copies of the lac repressor gene ensure high expression of the lac repressor, which binds to the operator region and lightly regulates expression of the recombinant exogenous peptide (His tag-C-terminal p53 palindromic tetramer-Antennapedia carrier). A Ni-nitrilotriacetic acid based column, provided as part of the kit, is used to purify the peptide through binding, washing and elution with appropriate buffers for the His tag. The final 21 kD peptide, with a PI of about 9.7, was easily purified from the total bacterial lysate by this approach (Figure 2).

For stable transfection into human cancer cell lines, the expression construct was incorporated into an inducible expression system in order to demonstrate the cytotoxic effects in a controlled manner. The MDA-MB-468 breast cancer cell line which expresses high levels of mutant p53 (Arg to His substitution at amino acid 273, one of the two most common p53 mutations in human cancers) was chosen as the model system. The tetracycline-inducible RevTet-On System (Clontech Laboratories, Palo Alto, CA) was used for these studies. Stably expressed reverse tetracycline-controlled transactivator (rtTA), under the control of a CMV promoter, was first established in the MDA-MB-468 cell line. Initially immunoblots with an antibody against the transactivator were used to identify high expression; subsequently a luciferase reporter system was used to identify highly inducible clones. This assay incorporated a luciferase reporter (Promega, Madison, WI) with the inducible pREvTRE vector of the RevTet-On system to measure the ability of tetracycline to induce luciferase activity. By means of these combined systems several MDA-MB-468 clones were identified which could express minimal luciferase activity without tetracycline exposure yet show high levels of luciferase activity after several hours in the presence of tetracycline. Using these clones, the inducible expression of the palindromic tetramer peptide was constructed in these cells. Additionally, the same inducible clones were used to express several control peptides, including the original single p53 C-terminal peptide, the jellyfish green fluorescent protein (GFP) and the palindromic tetramer fused to GFP. Since the anti-p53 antibody PAb-421 epitope is directed against p53 amino acids 371-380, which is contained in the peptide, measurement of expression of small amounts of the peptide in the inducible MDA-MB-468 model system via immunoblotting was possible. After 24 hours of exposure

of these cell sub-lines to the tetracycline analogue doxycycline, measurement of expression of the various peptides and/or the intensity and distribution of GFP was similarly possible.

Similarly, plasmid constructs suitable for expression via an adenovirus vector delivery system have been made for the C-terminal p53 peptide with and without Ant and for the tetrapeptide repeat with and without Ant, all with the GFP label. These plasmids are under the control of a CMV promoter (pCMV) and have an IRES segment separating the GFP from the peptide sequences so that both peptides and GFP are expressed simultaneously but independently (i.e., not covalently linked) so that expression can be confirmed by the GFP but the effect is due only to the peptide. The plasmid constructs tested for apoptotic activity thus include:

pCMV-IRES-GFP (control)
pCMV-p53Cterm-IRES-GFP (single repeat without Ant)
pCMV-p53Ant-IRES-GFP (single repeat with Ant)
pCMV-p53Ant-IRES-p53Ant (double dose of single repeat with Ant)
pCMV-4R-IRES-GFP (4 repeat without Ant)
pCMV-4RAnt-IRES-GFP (4 repeat with Ant).

These plasmids were packaged in an adenovirus vector system (Invitrogen, Carlsbad, CA) which was allowed to infect DU145 prostate cancer cells (with p53 mutations at 223 val->phe and 274 pro->leu) in culture at a multiplicity of infection of 50. The cells were assayed for apoptosis by propidium iodide staining after 48 hours.

B. Results

Effects of the C-Terminal p53 Palindromic Tetramer on Cells in Culture

In order to test the effect of the C-terminal p53 palindromic peptide cloned in E. Coli on cells in culture, the purified peptide was incubated at 30 μM with the human breast cancer cell line MDA-MB-468, and the effects compared to original monomer or single C-terminal peptide. Prior experiments had shown that exposure at this concentration was less than the IC_{50} for the original peptide. After exposure, the cells were collected, fixed in cold 70% ethanol, and the DNA was stained with propidium iodide. The DNA content was then quantified by flow cytometry in a FACSCalibur Cell Sorter (BD Bioscience, San Diego, CA). Cell cycle profiles were obtained, and the percentage of cells containing less than the diploid amount of DNA, indicative of cell undergoing cell death by apoptosis (sub-G1 peak), was quantified (Figure 3). Palindromic peptide treatment of MDA-468 at 30 uM for 6 hours showed 45% cell death whereas p53 C-terminal peptide (single repeat) showed 23% cell death (PI staining). This comparison shows that the C-terminal p53 palindromic peptide is about twice as effective in inducing apoptosis as the original single peptide sequence on an equimolar basis.

For the tetracycline-inducible peptide systems in MDA-MB-468 cells, cells were exposed to various concentrations of doxycycline for 24-48 hours and cell death measured by trypan blue exclusion (only dying cells are permeable to this dye) and propidium iodide staining (as above, in cells undergoing apoptosis DNA is cleaved so less PI can be bound resulting in accumulation of a sub-G1 peak of cells on flow cytometry). By trypan blue exclusion, control GFP cells showed approximately a 17% decrease in viability, cells expressing the single C-terminal sequence showed approximately a 30% decrease in viability, and cells expressing the p53 palindromic tetramer showed

greater than 80% decrease in viability. As measured by propidium iodide staining for the sub-G1 peak of cells on flow cyctometry, the P53 C-terminal 4-repeat cells, which stably express the 4-repeat under doxycycline regulation showed 43% cell death after induction whereas a control cell line, which inducibly expresses GFP showed background levels of 9%. By propidium iodide staining and flow cytometry, control GFP cells showed 12-15% apoptosis, cells expressing the single C-terminal sequence showed approximately 30% apoptosis, and cells expressing the p53 palindromic tetramer showed greater than 40% apoptosis (Figure 4). In the latter cells, there were also demonstrable changes in the expression of p53 responsive proteins, including an increase in the expression of the pro-apoptotic protein Bax (Figure 5), Fas and caspase 8, which is the initial caspase activated in the Fas death pathway (Figure 6). Interestingly, Bax expression from the 4 repeat is unique because the monomer, single p53 peptide, does not induce Bax, only the Fas related mechanism of apoptosis. This suggests that the 4 repeat can restore two targets of functional p53 where the monomer only restores one function. This implies a unique difference between the monomer and the 4 repeat.

Furthermore, using a specific inhibitor for caspase 8 eliminated the death induced by the C-terminal p53 palindromic tetrapeptide expression. The cleavage of the DNA repair signal protein poly-adenosyl-ribose polymerase (PARP), a key indicator of apoptosis, was also observed with the C-terminal p53 palindromic tetramer expression (Figures 5 and 6). Finally, one of the later events to occur as cells are undergoing apoptosis is the endonuclease cleavage of chromatin DNA at the DNA linker site between nucleosomes. These DNA fragments can be end-labeled with fluorescent-dUTP with the use of terminal

deoxynucleotidyl transferase. This technique, called TUNEL, is extremely sensitive for the detection of apoptosis. We used a commercial TUNEL assay (Medical & Biological Labs, Nagoya, Japan) with flow cytometry to examine these engineered cells. By this assay, 80% of the cells were killed with induction of the 4 repeat by doxycycline treatment (Figure 7). Furthermore, by using a specific inhibitor for caspase 8, IETD-FMK (Molecular & Biological Labs, Nagoya, Japan), death induced by the C-terminal p53 palindromic peptide expression (Figure 7) was eliminated. All of these findings support an apoptotic mechanism of cell death by this peptide.

It was also noted that the tetrapeptide has no to minimal effect on normal cells including the pluripotent stem cells for granulocytes, erythrocytes, monocytes and macrophages. It is taken up by all cells, but only kills cells that contain mutant p53.

Effects of C-Terminal p53 Palindromic Tetrapeptide (delivered as plasmid by adenovirus infection) on Cells in Culture

With adenoviral delivery, the control and various single repeat peptide plasmid constructs had minimal effect on DU145 cell viability, despite the fact that they were expressed as evidenced by the GFP label. On the other hand, the palindromic four repeat constructs caused >90% cell death (Figure 8). This was determined by western blotting to be due at least in part to the short half-life of the single repeat peptides in the cells. This could explain the differences in gene expression seen with the single repeat and four repeat peptides, in particular the fact that the former did not elicit Bax expression but the

latter did, and Bax expression is known to require a greater p53 activity. Also, the lack of any significant difference in effect between the single dose and double dose of the single repeat peptide suggests that dose alone is not as important as the fact the peptides in the four repeat are covalently linked and organized in such a fashion that they could mimic a natural p53 tetramer.

Effects of Expression of the C-Terminal p53 Palindromic Tetramer in Animals

As a first step in demonstrating the efficacy of the palindromic tetramer to induce apoptosis of cancer cells in vivo, inducible expression is tested in athymic nude mice. The MDA-MB-468 parent line has been shown to be tumorigenic in these mice due to their lack of a competent immune system.

Four groups of ten animals each are used. Groups 1 and 2 are injected with one million MDA-MB-468 cells containing the inducibly expressible palindromic tetramer in 0.5 ml of basement membrane media. Groups 3 and 4 are injected with one million MDA-MB-468 cells containing the inducibly expressible GKP. The tumors are allowed to develop to a measurable state (approximately 2-3 weeks). Then groups 1 and 3 are given 0.5 mg/ml doxycycline in their drinking water, and groups 2 and 4 are given drinking water without doxycycline. Tumor sizes are measured daily. Animals are then euthanized and the tumors removed for analysis. Tumor sections are assayed for levels of apoptosis by DNA cleavage assay (TUNEL) and activation of caspases and cleavage of PARP.

The palindromic tetramer is found to induce apoptosis of cancer cells in animals.

What is claimed is:

1. A polypeptide comprising a first segment of continuous amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1) covalently linked to a second segment of continuous amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2).

2. The polypeptide of claim 1, further comprising a glycine between the first segment and the second segment.

3. The polypeptide of claim 1, further comprising a six repeat histidine tag attached to the N-terminus of the polypeptide.

4. The polypeptide of claim 1, further comprising a membrane carrier peptide attached to the C-terminus of the polypeptide.

5. The polypeptide of claim 4, wherein the membrane carrier peptide (Ant) comprises amino acids having the sequence KKWKMRRNQFWVKVQRG (SEQ ID NO. 8).

6. The polypeptide of claim 1, further comprising:

   a. a six repeat histidine tag attached to the N-terminus of the polypeptide; and
   b. a membrane carrier peptide attached to the C-terminus of the polypeptide.

7. The polypeptide of claim 1, comprising amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD{glycine}DSDPGE

TKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 3), wherein the glycine may be present or absent.

8. The polypeptide of claim 1, comprising amino acids having the palindromic sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKE PGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 4), wherein the glycine may be present or absent.

9. The polypeptide of claim 7, comprising amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPGE TKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKEPGGSRA HSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPGETKFMLKKHRSTS QGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 5), wherein the glycine may be present or absent.

10. The polypeptide of claim 9, further comprising a six repeat histidine tag attached to the N-terminus of the polypeptide.

11. The polypeptide of claim 9, further comprising a membrane carrier peptide attached to the C-terminus of the polypeptide.

12. The polypeptide of claim 11, wherein the membrane carrier peptide comprises amino acids having the sequence KKWKMRRNQFWVKVQRG (SEQ ID NO. 8).

13. The polypeptide of claim 9, further comprising:

a. a six repeat histidine tag attached to the N-terminus of the polypeptide; and

b. a membrane carrier peptide attached to the C-terminus

of the polypeptide.

14. The polypeptide of claim 8, comprising amino acids having the palindromic sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKE PGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD[glycine]DSDPGETKFMLK KHRSTSQGKKSKLHSSHARSGGPEKGAQA[glycine]AQAGKEPGGSRAHSSHLK SKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 6), wherein the glycine may be present or absent.

15. The polypeptide of claim 14, further comprising a six repeat histidine tag attached to the N-terminus of the polypeptide.

16. The polypeptide of claim 14, further comprising a membrane carrier peptide attached to the C-terminus of the polypeptide.

17. The polypeptide of claim 16, wherein the membrane carrier peptide comprises amino acids having the sequence KKWKMRRNQFWVKVQRG (SEQ ID NO. 8).

18. The polypeptide of claim 14, further comprising:
    a. a six repeat histidine tag attached to the N-terminus of the polypeptide; and
    b. a membrane carrier peptide attached to the C-terminus of the polypeptide.

19. The polypeptide of claim 9, comprising amino acids having the palindromic sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSDDSDPGETKFMLKKHR STSQGKKSKLHSSHARSGGPEKGAQAAQAGKEPGGSRAHSSHLKSKKGQSTSRHKK LMFKTEGPDSDDSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 7).

20. A polypeptide comprising at least two covalently linked segments of continuous amino acids, each segment comprising consecutive amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1).

21. The polypeptide of claim 20, comprising three of the segments covalently linked.

22. The polypeptide of claim 20, comprising four of the segments covalently linked.

23. A polypeptide comprising at least two covalently linked segments of continuous amino acids, each segment comprising consecutive amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2).

24. The polypeptide of claim 23, comprising three of the segments covalently linked.

25. The polypeptide of claim 23, comprising four of the segments covalently linked.

26. A nucleic acid comprising nucleotides encoding the polypeptide of any one of claims 1-25.

27. A plasmid which expresses the polypeptide of any one of claims 1-25.

28. A method of killing cancer cells that contain mutant p53 or over-expressed wild-type p53 by contacting the cancer cells with the polypeptide of any one of claims 1-25.

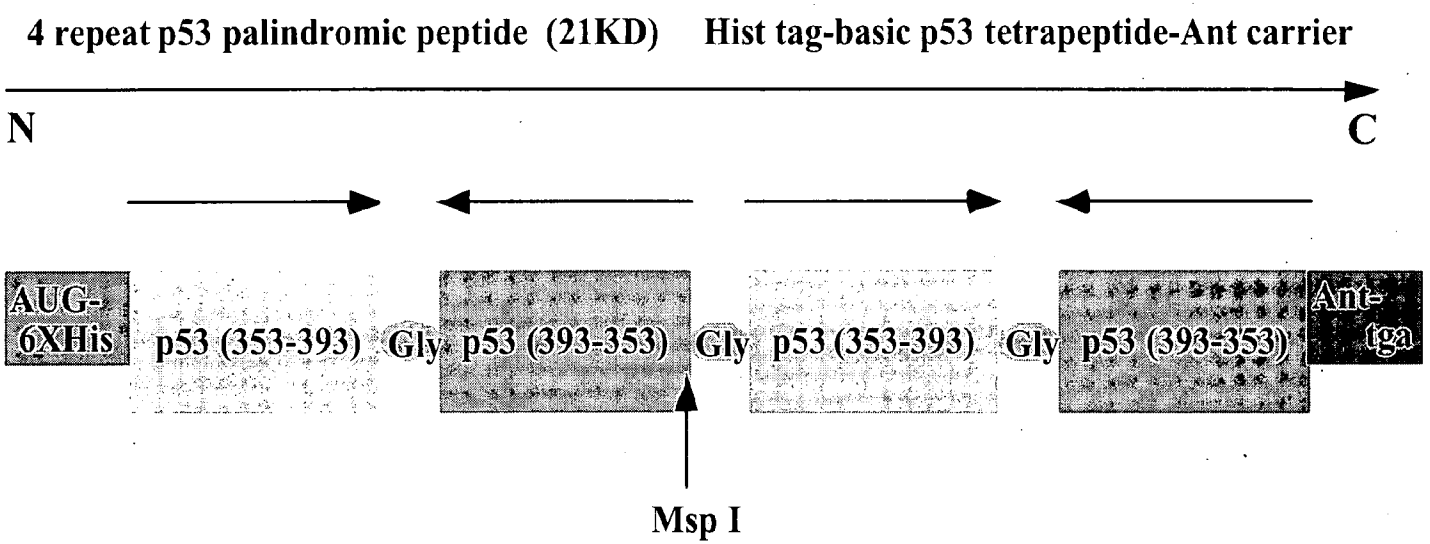
29. A method of treating a subject suffering from cancer by administering to the subject the polypeptide of any of claims 1-25.

C-TERMINAL p53 PALINDROMIC PEPTIDE THAT INDUCES APOPTOSIS OF CELLS WITH ABERRANT p53 AND USES THEREOF

Abstract of the Disclosure

Disclosed are polypeptides comprising a first segment of continuous amino acids having the sequence AQAGKEPGGSRAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD (SEQ ID NO. 1) covalently linked to a second segment of continuous amino acids having the sequence DSDPGETKFMLKKHRSTSQGKKSKLHSSHARSGGPEKGAQA (SEQ ID NO. 2), or at least two of each covalently linked to each other. The polypeptides are shown to induce apoptosis of cancer cells that contain mutant p53 or over-expressed wild-type p53.

FIGURE 1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AUG-6XHis
p53 (353-393)
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p53 (393-353)
Gly
p53 (353-393)
Gly
p53 (393-353)
Ant-tga
Msp I

FIGURE 2

FIGURE 3

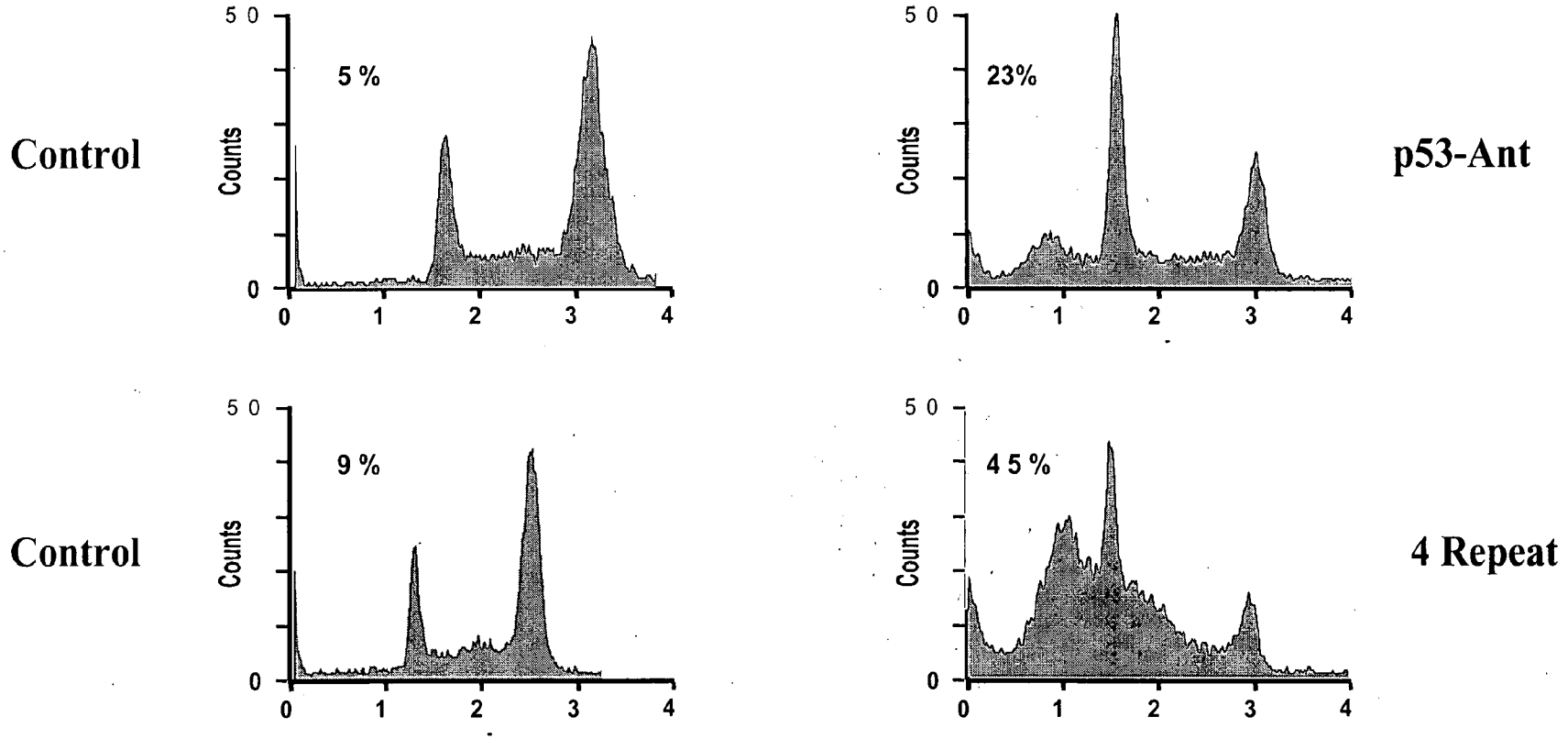
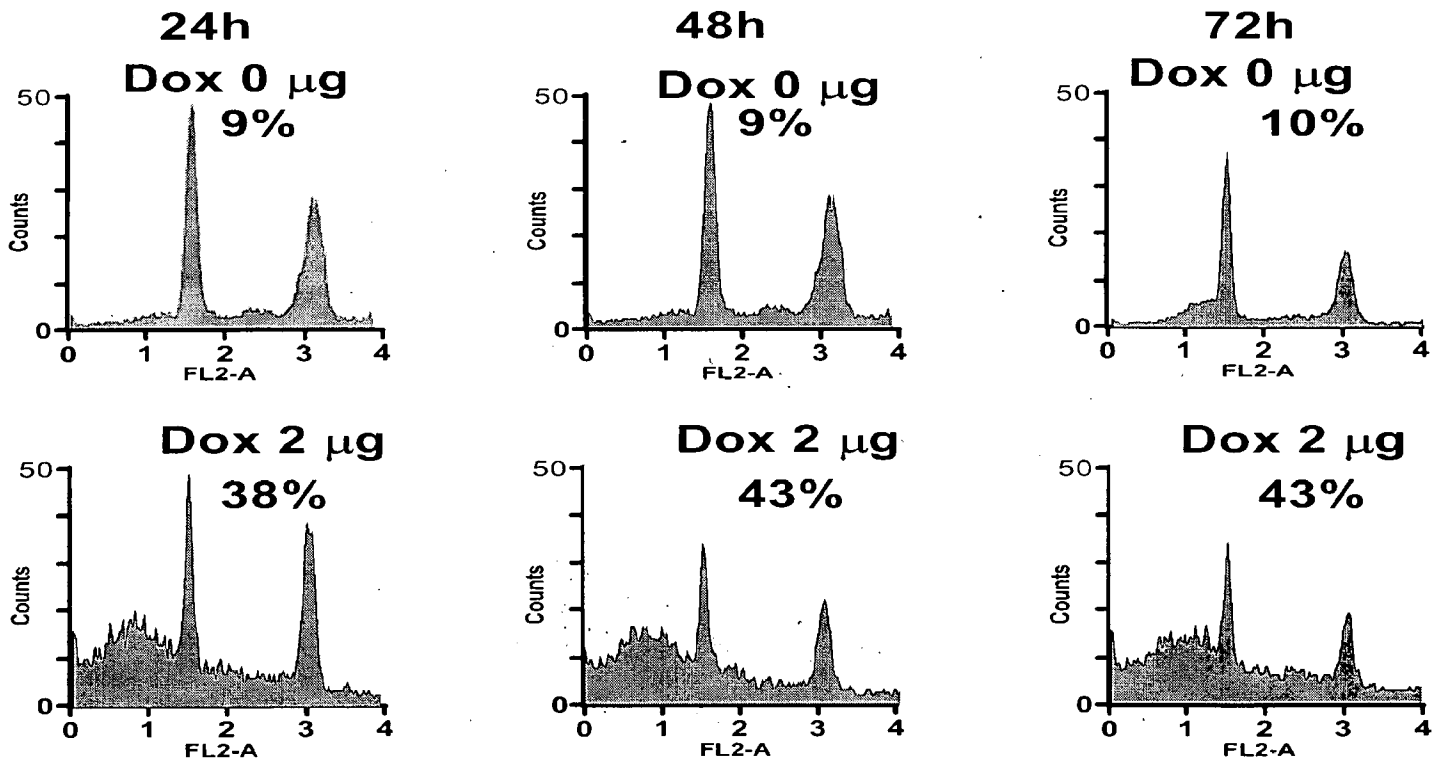


FIGURE 4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Dox 0 µg
9%
48h
Dox 0 µg
9%
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10%
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43%
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43%
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FL2-A
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2
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4
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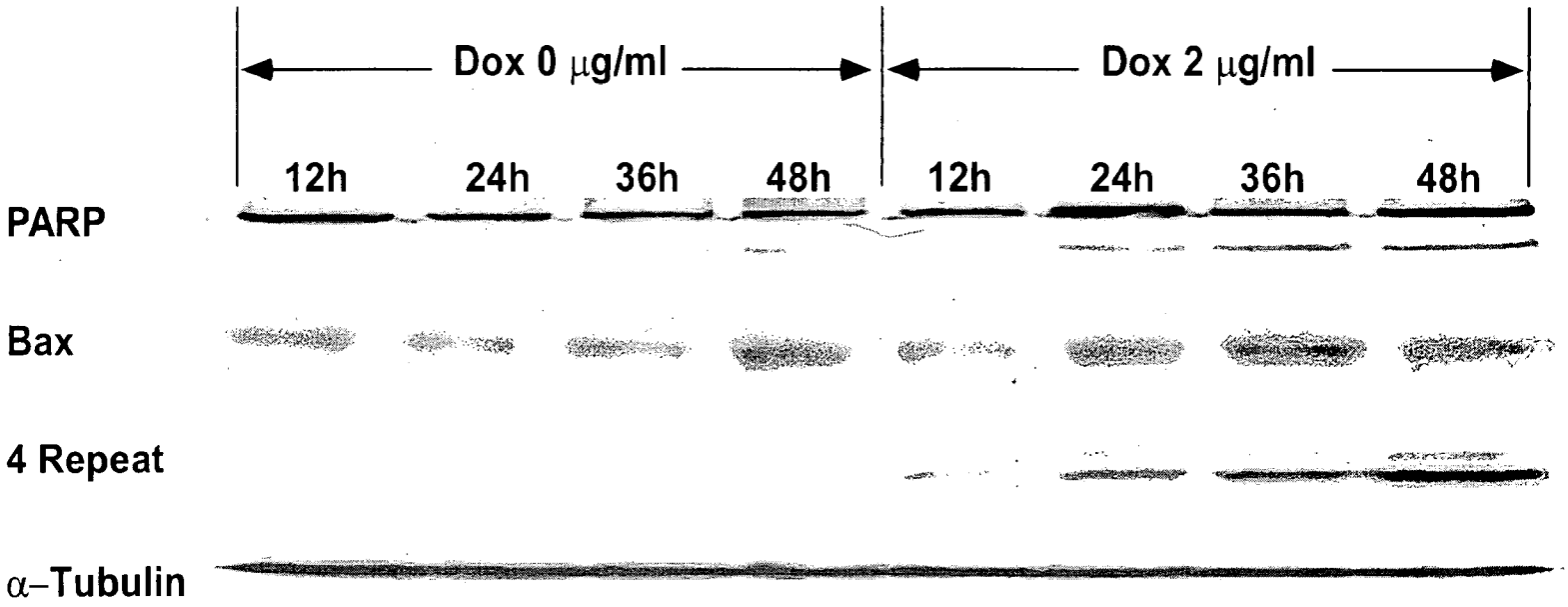
FIGURE 5



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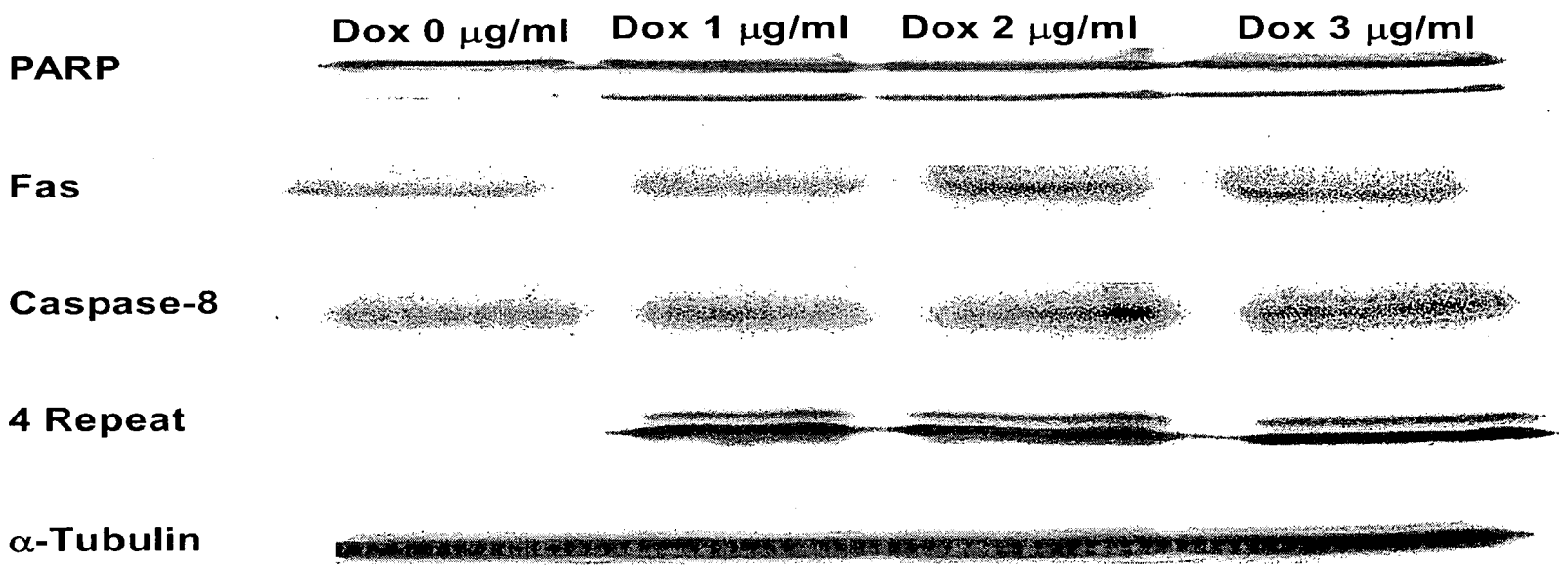

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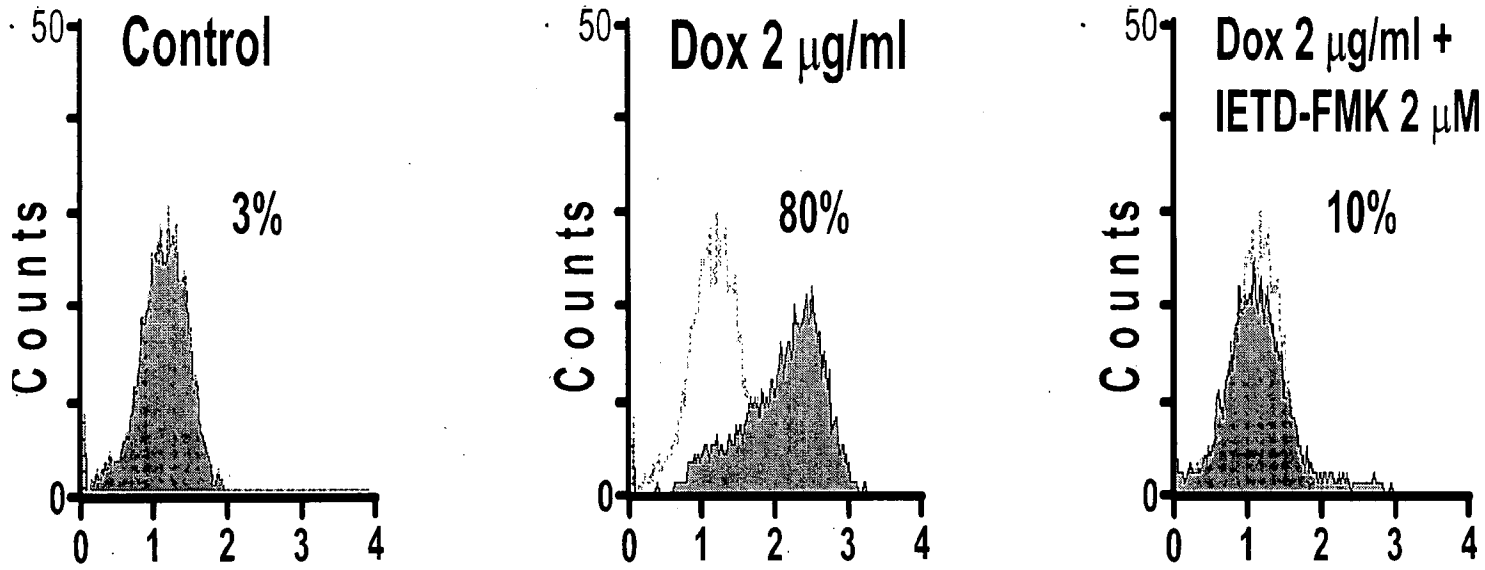
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Dox 2 μg/ml +
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Counts
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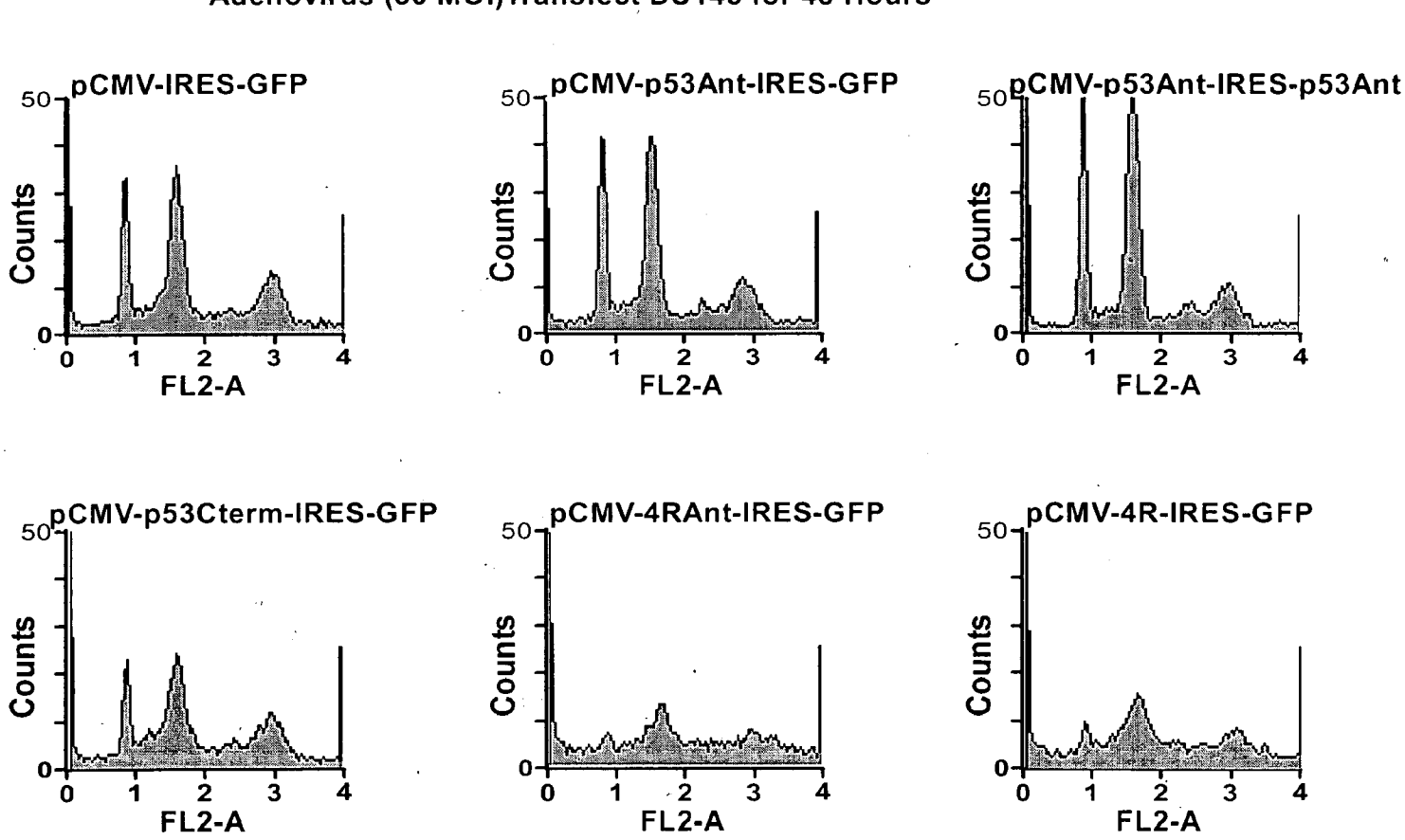
0
0 1 2 3 4

FIGURE 8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SEQUENCE LISTING

<110> Fine, Robert

Brandt-Rauf, Paul

Mao, Yueha

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Gln Ser Thr Ser Arg His Lys Lys Leu Met Phe Lys Thr Glu Gly Pro
65                  70                  75                  80

Asp Ser Asp Gly Asp Ser Asp Pro Gly Glu Thr Lys Phe Met Leu Lys
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20 25 30

Phe Lys Thr Glu Gly Pro Asp Ser Asp Asp Ser Asp Pro Gly Glu Thr
35 40 45

Lys Phe Met Leu Lys Lys His Arg Ser Thr Ser Gln Gly Lys Lys Ser
50 55 60

Lys Leu His Ser Ser His Ala Arg Ser Gly Gly Pro Glu Lys Gly Ala
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Gln Ala Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser
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Ser His Leu Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys
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Leu Met Phe Lys Thr Glu Gly Pro Asp Ser Asp Asp Ser Asp Pro Gly
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